UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC EFFECTS OF METHYLGERAMBULLIN AND BIS (METHYLTHIOMETHYL)-DISULPHIDE (SB) ON T-LYMPHOBLASTIC LEUKEMIC CELL LINE (CEM-SS)

SHAR MARIAM MOHAMED

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By

SHAR MARIAM MOHAMED

Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

August 2000

DEDICATION

Dedicated to my dear husband, I cannot thank you enough for your love and support. Having the blessings of being your wife is certainly my wildest dream come true. Your incredibly unique love has touched me in ways I cannot describe and I am deeply thankful (syukur) to the Almighty that I've been given the chance to weave my love with yours. My deepest gratitude and love to my dear parents Mohamed Hj. Ismail and Hasnah Midon, sister Ayu and brother Don for their everlasting eternal support, courage and love, which keep me going as a stronger person each day. I am always with you more than ever. To my in-laws, I am so blessed with your warmth and support……thank you.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chairman: **Associate Professor Abdul Manaf Ali, Ph.D.**

Faculty: **Food Science and Biotechnology**

The cytotoxic effects of 2 sulphur-containing compounds were studied on T-lymphoblastic leukemic cell line. Methylgerambullin is believed to be a new sulphone derived from a methylthiopropenoic acid isolated from *Glycosmis calcicola* (family *Rutaceae).* Another sulphonic compound is bis-(methylthiomethyl)-disulphide, an extract from *Scorodocarpus borneensis* (family *Olacaceae*) with irritating garlic-like odor. Cytotoxic activities of methylgerambullin and bis-(methylthiomethyl)-disulphide were tested against CEM-SS (T-lymphoblastic leukaemia), KU812F (chronic myelogeneous leukaemia), UACC-62 (melanoma) and HT29 (colon cancer) cell lines using MTT, a colorimetric tetrazolium-based assay. Cytotoxic concentrations of the compounds that killed cells by 50% (CD50) with respect to untreated cell population, varied among the cell lines tested. CEM-SS was found to be the most sensitive cell line to methylgerambullin and bis-(methylthiomethyl)-disulphide with CD50 = 0.25 µg/ml and 3.50 µg/ml respectively. The cytotoxic effects exerted by both compounds on this cell line was studied from both morphological

manner over 72 hours period. Microscopic observations, including inverted microscopy of live cultures, fluorescent microscopy of acridine orange-propidium iodide stained cultures, and scanning and transmission electron microscopy showed that both necrotic and apoptotic death occurred in methylgerambullin- and bis-(methylthiomethyl)-disulphide-treated cell populations, based on morphological criteria. From agarose gel electrophoresis and quantitative analyses of internucleosomal cleavage, treatments with these compounds at their respective CD50 doses did not yield random or multiple of 180-200 bp DNA fragmentation which often associated with necrotic and apoptotic deaths respectively. Such observation may simply owe to the fact that the percentage of apoptosis and necrosis events were fairly low as quantified after acridine orange-propidium iodide staining, or may also suggest the involvement of sulphur residue in methylgerambullin and bis-(methylthiomethyl)-disulphide which act as an antioxidant, thus protecting DNA degradation from occuring. Flow cytometric analyses based on annexin V-FITC (fluorescein isothiocynate) binding to the phosphatidylserines residue which was translocated from the inner to the outer leaflet of the plasma membrane showed that the onset of apoptosis in both methylgerambullin- and bis-(methylthiomethyl)-disulphide-treated population was at 6 hours exposure. Both methylgerambullin and bis-(methylthiomethyl)-disulphide induced G0/G1 arrest up to 48 hours and 24 hours respectively followed by arrest in the subsequent S phase.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

KESAN SITOTOKSIK OLEH METHYLGERAMBULLIN AND BIS-(METHYLTHIOMETHYL)-DISULPHIDE (SB) KE ATAS SEL-SEL T-LIMFOBLASTIK LEUKEMIA (CEM-SS)

Oleh

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Ogos 2000

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Kesan sitotoksik oleh 2 sebatian yang mengandungi sulfur telah dikaji ke atas sel-sel T-limfoblastik leukemia. Metilgerambullin, dipercayai satu sebatian sulfon yang terbaru ditemui adalah hasilan terbitan daripada asid metiltiopropenoik yang diekstrak daripada *Glycosmis calcicola* (keluarga *Rutaceae).* Satu lagi sebatian ialah bis-(metiltiometil)-disulfida yang diekstrak daripada *Scorodocarpus borneensis* (keluarga *Olacaceae*) yang berbau seperti bawang putih. Aktiviti sitotoksik metilgerambullin dan bis-(metiltiometil)-disulfida telah diuji ke atas sel-sel CEM-SS (T-limfoblastik leukemia), KU812F (kronik myelogeneous leukemia), UACC-62 (melanoma) dan HT29 (kanser kolon) menggunakan kaedah MTT yang bergantung kepada perubahan warna tetrazolium. Hasil kajian menunjukkan bahawa sel CEM-SS adalah sel yang paling sensitif terhadap metilgerambullin dan bis-(metiltiometil)-disulfida dengan kepekatan yang berupaya membunuh 50% daripada populasi sel (CD50) iaitu 0.25 μg/ml bagi metilgerambullin dan 3.50 μg/ml bagi bis-(metiltiometil)-disulfida. Kesan sitotoksik sebatian-sebatian ini dinilai melalui perubahan pada morfologi sel dan

perubahan di peringkat molekular selepas rawatan dengan sebatian-sebatian tersebut selama 72 jam. Penilaian mikroskopik termasuklah menggunakan mikroskop terbalikan ke atas kultura hidup, mikroskop floresen selepas pewarnaan sel dengan akridin oren- propidium iodida dan mikroskopi elektron menunjukkan bahawa kematian apoptosis dan nekrosis berlaku di dalam populasi sel yang dirawat dengan metilgerambullin dan bis-(metiltiometil)-disulfida. Daripada keputusan yang diperolehi melalui elektroforesis pada jel agaros dan analisis kuantitatif pada belahan nukleosom, rawatan dengan kedua-dua sebatian pada dos CD50 masing-masing tidak menghasilkan belahan rawak atau belahan spesifik 180-200 bp pada DNA yang masing-masing dikaitkan dengan kematian nekrosis dan apoptosis. Pemerhatian tersebut mungkin disebabkan oleh peratusan kematian apoptosis dan nekrosis yang terlalu sedikit sepertimana yang telah dikenalpasti secara kuatitatif melalui kaedah pewarnaan sel dengan akridin oren dan propidium iodida, atau mungkin disebabkan oleh kesan perlindungan sulfur yang terdapat pada methylgerambullin dan bis-(metiltiometil)-disulfida yang bertindak sebagai antioksidan yang melindungi daripada berlakunya belahan DNA. Analisis menggunakan flow saitometer berdasarkan pada ikatan annexin V-FITC (fluoresen isotiosianida) pada fosfatidilserine yang mengalami translokasi dari lapisan dalam ke lapisan luar membran plasma, menunjukkan bahawa kejadian apoptosis dalam populasi sel yang dirawat dengan methylgerambullin dan SB berlaku seawal 6 jam selepas rawatan. Methylgerambullin dan SB juga didapati berupaya merencatkan sel pada fasa G0/G1 dalam kitaran sel hingga 48 jam dan 24 jam masing-masing selepas rawatan, diikuti dengan rencatan pasa fasa S dalam kitaran sel.

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upset once in a while when the contaminants were not discarded properly). To have the wonderful opportunity to work with you guys and to experience the enjoyable moments is an everlasting souvenir that I will always cherish.

I certify that an Examination Committee met on 28 August 2000 to conduct the final examination of Shar Mariam Mohamed on her Doctor of Philosophy thesis entitled "Cytotoxic Effects of Methylgerambullin and Bis-(methylthiomethyl)-disulphide (SB) on T-Lymphoblastic Leukemic Cell Line (CEM-SS)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfilment of the requirements for the degree of Doctor of Philosophy.

KAMIS AWANG, Ph.D,
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Date: **14 DEC 2000**

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Candidate
SHAR MARIAM MOHAMED

Date: 25 October 2000

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LIST OF ABBREVIATIONS

2-CDA ..... chloro deoxyadenosine
ABTS. ..... 2,2'-Azino-di[3-ethylbenzthiazolin-sulfonat]
ADP... ..... adenosine diposphate
AIF..... ..... apoptosis indusing factor
ALL ... . acute lymphoblastic leukemia
AML .. .. acute myeloid leukemia
AO .... acridine orange
Ara-c.. ..... arabinofuranosylcytosine
ATP ... . adenosine triposphate
CA .... calyculin A
CD50 . ..... cytotoxic dose resulting in 50% reduction of cell population
CGM.. ..... complete growth medium
CLL.... ..... chronic lymphocytic leukemia
CML .. .. chronic myeloid leukemia
CO^2.... ..... carbon dioxide
CV .... coefficient of variation
Cyt-c .. .. cytochrome c
DCF ... . deoxycorfomycin
DDS... ..... diaminodiphenyl sulphone
DMSO ..... dimethyl sulfoxide
DNA .. .. deoxyribonucleic acid
ER..... ..... endoplasmic reticulum
FDA... ..... Food and Drug Administration
FITC .. .. fluorescein isothiocyanate
FSC.... ..... forward scatter
GSH... ..... glutathione
HCl .... hydrochloric acid
HIV.... ..... human immunodeficiency virus
ICAD . ..... inhibitor of caspase-activated DNAse
ICE..... ..... interleukin converting enzyme
KCl .... potassium chloride
MTT .. .. 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTA .. .. microculture tetrazolium assay
MTS... ..... 3-[4,5-dimethylthiazol-2-yl]-5-(carboxymethoxyphenyl),(4-sulphophenyl)-2H-tetrazolium
MeOH ..... methanol
MTP... ..... microtiter plate
NaCl .. .. sodium chloride
NHAC N4-hexadecyl-1-β-D-arabinofuranosylcytosine
OD .... optical density
OA .... okadaic acid
PARP. ..... poly(ADP-ribose) polymerase
PBMC ..... peripheral blood mononuclear cell
PBS.... ..... phosphate buffered saline
PI .... propidium iodide
PMS... ..... phenazine methosulphate

PMT ... . photo multiplier
PS..... ..... phosphotidylserine
PT .... permeability transition
RNA... ..... ribonucleic acid
RNP ... . ribonucleoprotein particles
ROS ... . reactive oxygen species
SB .... bis-(methylthiomethyl)-disulphide isolated from *Scorodocarpus borneensis* Becc.
SDS.... ..... sodium dodecyl sulphate
SEM... ..... scanning electron microscope
SSC.... ..... side scatter
TEM .. .. transmission electron microscope
TNF ... . tumor necrosis factor
TUNEL..... Tdt-mediated dUTP-biotin nick end labelling
UV .... ultraviolet
XTT ... . sodium (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide)

CHAPTER I

INTRODUCTION

Screening for potential anticancer compounds has been one of the most important aspects in cancer research. Many commercially available drugs are synthetically derived, for example etoposide, a semi-synthetic epipodophyllotoxin that is one of the most commonly used anti-cancer drugs (Joel, 1996). Although these synthetic drugs are known to be very effective against cancer cells, they also possess some undesirable side effects such as neurotoxicity, fibrinolysis and ototoxicity (hearing loss) (Casciato and Lowitz, 1995). Thus, choices for improvement are confined to introduce natural products as potential anticancer agents. Phytochemicals, one of these natural substances have been known to exhibit a wide variety of medicinal value ever since the ancient times. The scientific theories underlying their medicinal properties however remained unknown then. Wilkinson, (1998) recently highlighted that herbal products have great potential in the emerging nutraceutical and pharmaceutical industries in that they are widely consumed as food and are used in preventive and curative treatments throughout the world. It was reported in the same study that tropical forests have produced 47 major pharmaceutical drugs of world-wide importance and it is estimated that 328 potential drugs of major importance is yet to be discovered which would be worth $147 billion. The tropical forests still offers 125,000 flowering plant species that are of pharmacological relevance but this will involve 50,000 to one million screening

tests to discover one profitable drug, which seem to be the ultimate constraint in the discovery of new drugs.

It has been reported by the Alliance Pharmaceutical Corporation that on average, 12 years are needed for an experimental drug to be brought from the laboratory to the market shelf (Wierenga and Eaton, Internet). Preclinical testing requires three and a half years of experiments in laboratory on *in vitro* and animal models to assess safety and biological activities of the compound. Another six years are required for clinical trials that assess the safety, dosage, effectiveness and monitor the adverse reaction of the compound. Only five in 5000 compounds that enter preclinical testing make it to human testing. Approval process by the Food and Drug Administration (FDA) will take about two and a half years where normally only one of the five compounds tested in humans is approved.

Despite the duration of time required to evaluate a potential drug, numerous phytochemicals have been scientifically isolated and their discovery was very much directed in the perspective of anticancer drug development. Plant constituents such as taxol, camptothecin, podophyllotoxin, vinblastine and vincristine represent some of the most important drugs currently utilized for the treatment of human cancers (Wall and Wani, 1993). Numerous more phytochemicals are under investigations and have the potential to be on the pharmaceutical market shelves. Among these are betulinic acid, gonoithalamin and dammarane-type triterpenes, to name a few. Betulinic acid, which is available in abundant supply from the bark of white birch trees was reported to

be specific for inhibiting human melanomas (Pisha *et al.*, 1995). Gonoiothalamin, isolated from various *Gonoiothalamus sp.* was found to be toxic to cervical, pancreas, gastric and breast carcinoma (Ali *et al.*,1996). Dammarane-type triterpenes from *Cloeme africana* has been shown to be highly toxic against leukemia (Nagaya *et al.*, 1997).

One of the most extensively studied diseases is leukemia that remains to be a formidable disease. Due to the ease of obtaining repeated samples from blood, marrow or lymph nodes, most of the principles of cancer therapy is largely based on the leukemia model. Important breakthroughs in cancer therapy were achieved in the treatment of leukemia, which includes the use of combination therapy, immunotherapy, supportive therapy using antibiotics and blood product support and bone marrow transplant (Ng, 1996). Treatment with antileukaemic drugs however only controls the symptom without enhancing the survival rate (Arthur, 1989). Chemotherapeutic agents, also known as cytotoxins due to its ability to kill cells are normally less specific in that these agents kill not only cancer cell but also normal cells. Thus, the most apparent implication of chemotherapy is emanation of side effects which include hair loss, nausea, vomitting, constipation and can be as disastrous as myelosuppression (drop in blood count – neutropenia, thrombocytopenia, anaemia), and damage on cardiac, pulmonary, renal and hepatic systems (Ng, 1996). This prompted efforts to screen for antileukaemic agents that will have a dramatic cytotoxic effect specifically on the target cells. Most of the drugs currently used act via two distinct mechanisms: inhibition of cell proliferation or induction of active cell

death (Vial *et al.*, 1997). Compounds that act via the latter mechanism are likely to be a better candidate as an antileukaemic agent because in the former action, some individual cells may have escaped through salvage pathways, and will continue to proliferate. The advancement in research techniques and the introduction of new molecular tools in recent years have prompted more efforts in investigations to build understanding on how exposure to the existing or newly discovered drugs lead to cell death. The efficacy of these drugs has been critically assessed by the many mechanisms of cell death and resistance that adversely limit their efficacy. Today, due to the urgent need for studies in various fields that closely mimics the animal systems, animal cell culture which was only an esoteric art during those time has become an established technology. New drug discovery, for example, may not be recognized and may be unsafe for use in man. Thus, pre-clinical trials are needed, which can be made possible with cell culture technology.

It is thus crucial to determine the mode of action exerted by a potential anticancer agent. This has led to the design of the experimental approach which will be implemented in the current study. Both cellular and molecular changes in treated cell populations will be examined and compared with control (untreated) populations in a time course manner. Hence, either necrotic or apoptotic cell death could be suggested. Morphological studies may offer answers to whether physiological and accidental deaths are causally related and to the degree of overlapping between apoptotic and necrotic pathways.